HHS Public Access

Author manuscript

Semin Arthritis Rheum. Author manuscript; available in PMC 2023 October 01.

Published in final edited form as:

Semin Arthritis Rheum. 2022 October ; 56: 152052. doi:10.1016/j.semarthrit.2022.152052.

False positive anti-Topoisomerase I (Scl-70) antibody results in clinical practice: A case series from a scleroderma referral center

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Abstract

Purpose: To determine if some patients who tested positive for anti-Scl-70 antibody in clinical practice, but did not have classifiable systemic sclerosis, were negative for anti-Scl-70 antibody by the more specific immunodiffusion method of testing.

Methods: Patients evaluated by a rheumatologist at a Scleroderma referral center who had tested positive for anti-Scl-70 antibody prior to referral, but did not have classifiable SSc based on clinical criteria, were invited to undergo testing for anti-Scl-70 antibody by immunodiffusion. Patient demographics and clinical features were recorded at the time of their evaluation, and diagnostic testing results were reviewed using the medical records.

Results: 52 patients were enrolled over an 8-year period, with 48 (92.3%) testing negative and 4 (7.7%) testing positive for anti-Scl-70 antibody by immunodiffusion. Of the 48 patients who tested negative, 18 (37.5%) tested negative for ANA by indirect immunofluorescence, 33 (68.8%) did not have Raynaud's phenomenon, and 43 (89.6%) had ≤1 clinical criteria items based on the 2013 ACR/EULAR SSc classification criteria. Nevertheless, 21 (43.8%) patients who were

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CRediT authorship contribution statement

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.semarthrit.2022.152052.

negative for anti-Scl-70 antibody by immunodiffusion had undergone a chest CT and 14 (29.2%) had undergone an echocardiogram. A total of 23 patients had at least one follow up clinic visit. 3 out of 4 patients who were positive for anti-Scl-70 antibody by immunodiffusion, but none of the 19 patients who tested negative by immunodiffusion, developed sufficient criteria during follow up to be classified as SSc.

Conclusion: Assays for anti-Scl-70 antibody in commercial laboratories that are commonly utilized in clinical practice can produce false positive results. These results can lead to angst for patients, as well as unnecessary referrals and diagnostic evaluations.

Keywords

Systemic sclerosis; Scleroderma; Scl-70 antibody; Topoisomerase I antibody; ANA; Immunodiffusion

Introduction

Systemic sclerosis (SSc) is an autoimmune disease with heterogeneous clinical manifestations [1]. Autoantibody tests are widely used to aid in the diagnosis of SSc, and also have prognostic significance [2]. Anti-Scl-70 antibody (also known as anti-topoisomerase I antibody), one of three autoantibodies included in the 2013 ACR/EULAR classification criteria for SSc [3], has been shown to be specific for SSc and portends a risk of diffuse skin involvement, progressive interstitial lung disease (ILD), and mortality [4–12].

Anti-Scl-70 antibody in SSc patients was first described in 1979, after antibodies from some SSc patients were found to react with a 70-kilodalton protein purified from rat liver nuclei [13]. The Scl-70 antigen (originally named based on its molecular weight) was subsequently identified as a soluble proteolytic product of DNA Topoisomerase I [14–16]. The Scl-70 antigen can be enriched from rabbit or calf thymus and serve as the target for detection of anti-Scl-70 antibody in serum samples via passive immunodiffusion [14,17]. Besides immunodiffusion, several alternative assays for detection of anti-Scl-70 antibody have been described. These include enzyme-linked immunosorbent assay (ELISA) (sometimes referred to as enzyme immunoassay or EIA), immunoblotting, counterimmunoelectrophoresis (CIE), immunoprecipitation, chemiluminescent immunoassay (CIA), line blot immunoassay (LIA), and multiplex flow immunoassay using fluorescently-dyed beads coated with antigens (sometimes referred to as multiplex bead assay or multiple-bead assay) [5,14,18–24]. Adding another source of potential variation, recombinant Topoisomerase I protein can be utilized as the target for anti-Scl-70 antibody detection instead of native Topoisomerase I enriched from rabbit or calf thymus [25].

Practicing clinicians depend on commercial or local clinic or hospital laboratories for autoantibody testing, including for anti-Scl-70 antibody. However, the extent to which the anti-Scl-70 antibody testing methods employed by each laboratory have been validated for use in the diagnostic evaluation for SSc is unclear. A recent report based on data from an academic laboratory showed that anti-Scl-70 antibody testing by a multiplex flow immunoassay had poor specificity for SSc [26]. ELISA showed better specificity than the multiplex flow immunoassay, but substantially poorer than that of immunodiffusion. Poor

specificity of a multiplex flow immunoassay and an ELISA, which are two assay formats commonly offered by commercial laboratories in the U.S. for anti-Scl-70 antibody testing, raises concern that false positive anti-Scl-70 antibody results could be widespread in clinical practice. Here we present a series of cases that validates this concern, by identifying patients who had tested positive for anti-Scl-70 antibody in a commercial or local laboratory but who did not have SSc and tested negative for anti-Scl-70 antibody by immunodiffusion.

Methods

Patient selection

Each patient had been referred to the Rheumatology clinic at the University of Texas Health Science Center-Houston (UTHSC—H), and was evaluated by one of four rheumatologists (SA, BJ, MDM, or BS). Patients were invited to participate in this study if they had the following characteristics: (1) had tested positive for anti-Scl-70 antibody performed by a commercial or local laboratory prior to their evaluation in our clinic, and (2) did not have sufficient clinical criteria, at the time of our initial evaluation, to be classified as SSc according to the 2013 ACR/EULAR criteria [3]. Of note, since we hypothesized that some patients' commercial anti-Scl-70 antibody results could be falsely positive, commercial anti-Scl-70 antibody results were not counted as positive in the patients' classification criteria for this study. For example, a patient with Raynaud's phenomenon but no other clinical criteria was given a score of 3 rather than 6. Therefore, patients with 6–8 points based on clinical criteria alone (not counting their commercial anti-Scl-70 antibody result) were also invited to enroll. Two patients' scores fell within this range; these two patients are each discussed in more detail in the Results section. All patients provided informed consent. This study was approved by the UTHSC—H Institutional Review Board Committee for the Protection of Human Subjects.

Clinical and diagnostic data

Demographic and clinical information was collected at the time of the patient's initial evaluation in our clinic. SSc criteria are reported based on the patient's initial evaluation in our clinic, but were also recorded for each patient's follow up visits to determine whether or not patients developed additional criteria over time. The follow up time was recorded as the months from initial to most recent evaluation in our clinic. History of Raynaud's phenomenon and physical exam findings were based on the judgment of the evaluating rheumatologist, adhering to the item definitions in the 2013 ACR/EULAR criteria [3]. Diagnostic tests including antinuclear antibody (ANA), chest CT, and echocardiogram were reviewed using the patients' medical records. ANA by indirect immunofluorescence was considered positive in this study if the titer was ≥1:80. For the three patients who did not have an ANA result by indirect immunofluorescence available in their medical records, ANA was determined in our research laboratory, using serum at 1:80 dilution and reagents from ZEUS Scientific (REF FA2400), following the manufacturer's instructions. The determination of interstitial lung disease (ILD) was based on radiology reports from chest CT's found in the medical records; findings counted as positive for ILD were ground glass opacities, subpleural reticulation, traction bronchiectasis, honeycombing, or explicit mention of NSIP, UIP, ILD, or fibrosis. Echocardiogram reports were reviewed to determine

whether or not there was evidence of right ventricular systolic dysfunction or an estimated right ventricular systolic pressure (RVSP) >35 mmHg. Of note, only one patient in this study had undergone right heart catheterization.

Commercial and local laboratory information

Each patient's commercial or local laboratory anti-Scl-70 antibody result was reviewed for the titer, reference range, and company or location that performed the testing. Each commercial laboratory's method(s) of anti-Scl-70 antibody testing, and whether or not they offer immunodiffusion testing for anti-Scl-70 antibody, was queried via search of that company's website and via direct online and phone inquiries by BS. The information on method(s) of testing offered by each company via their website and/or response to our inquiry is reported. Local clinic or hospital systems from which anti-Scl-70 antibody results were found in this study were not contacted, but their reported titers and reference ranges are reported.

Anti-Scl-70 antibody testing by immunodiffusion

Patients underwent testing for anti-Scl-70 antibody via immunodiffusion in our Division's research laboratory. Serum was prepared from peripheral blood samples using standard methodology and stored at −80 °C, then thawed to room temperature on the day of testing. Undiluted serum and Scl-70 antigen were subjected to passive immunodiffusion, using reagents from Inova Diagnostics (REF 708475). Each gel plate included one Scl-70 antibody-positive control. Samples were incubated for three days, then evaluated for precipitin lines between serum wells and the Scl-70 antigen well.

Results

52 patients were included over an 8-year period from 2013 to 2021. 48 of these patients tested negative for anti-Scl-70 antibody by immunodiffusion, while 4 patients tested positive. An example of anti-Scl-70 antibody testing results is shown in Fig. 1.

Demographic, clinical, and diagnostic information

Summary demographic, clinical, and diagnostic information are shown in Table 1. Individual patient data are shown in Appendix A.

Patients positive for anti-Scl-70 antibody by immunodiffusion

Of the four patients who were confirmed as positive for anti-Scl-70 antibody by immunodiffusion, three patients had at least two SSc criteria items at initial visit based on history and physical exam (Raynaud's phenomenon, abnormal nailfold capillaries, puffy fingers, and/or fingertip pitting scars). One patient with 8 points in the ACR/EULAR classification criteria based on clinical features alone was classifiable as SSc once positivity for anti-Scl-70 antibody was confirmed by immunodiffusion. Two other patients who did not have sufficient criteria to be classified as SSc during their initial clinic visit developed additional SSc features sufficient to be classified as SSc during follow up. Two patients who had not had ILD on initial screening chest CT subsequently developed ILD during follow up.

One patient was found to be positive for anti-Scl-70 antibody during evaluation for pulmonary hypertension, but had no Raynaud's or cutaneous features of SSc. Her forced vital capacity (FVC) was normal, and chest CT did not show evidence of ILD. Right heart catheterization revealed an elevated mean pulmonary arterial pressure of 30 mmHg, but with an elevated pulmonary capillary wedge pressure of 17 mmHg, consistent with WHO Group 2 pulmonary hypertension. The significance of the positive anti-Scl-70 antibody in her case is unclear.

Patients negative for anti-Scl-70 antibody by immunodiffusion

44 (91.2%) of the patients who tested negative for anti-Scl-70 antibody by immunodiffusion were female, and the mean age at enrollment was 45.5 years.

Only 15 of these 48 patients (31.3%) had experienced Raynaud's phenomenon, more than half had no clinical SSc criteria, and 43 (89.6%) had ≤1 SSc criteria items. Additionally, 18 of these 48 patients (37.5%) tested negative for ANA by indirect immunofluorescence.

A large percentage of patients had undergone more extensive diagnostic evaluation prior to referral, including 14 (29.2%) who underwent an echocardiogram and 21 (43.8%) who underwent a chest CT. The majority of these studies were performed on patients who had ≤1 SSc criteria items (Fig. 2 and Appendix A).

Regarding the two patients with evidence of ILD on chest CT, the finding of ILD had preceded autoantibody testing in both cases. One was an 80 year-old man with a history of smoking and a radiographic pattern suggestive of usual interstitial pneumonia (UIP)—a presentation suggestive of idiopathic pulmonary fibrosis (IPF). The commercially positive anti-Scl-70 antibody result was part of a screening performed by his Pulmonologist during work-up for the etiology of ILD. Hisecho showed an elevated estimated RVSP but normal RV systolic function, and to date he has not undergone right heart catheterization. He had not experienced Raynaud's phenomenon, and he had no cutaneous exam findings suggestive of SSc. The other patient with ILD was a 44 year-old woman who had developed ILD in her 30′s, then subsequently developed Raynaud's phenomenon, and had abnormal nailfold capillaries on exam. She had no other cutaneous features of SSc, and tested negative for ANA by indirect immunofluorescence. Her chest CT radiology report noted pulmonary fibrosis with "fine honeycombing" and interlobular septal thickening but did not specify a radiographic pattern. The other 19 patients who were negative for anti-Scl-70 antibody by immunodiffusion and underwent chest CT did not have evidence of ILD.

19 patients had at least one follow up visit, with a median interval of six months. None of these 19 patients developed SSc during follow up.

Commercial and local anti-Scl-70 antibody results

For the 48 patients whose anti-Scl-70 antibody result by immunodiffusion was negative, the laboratories in which they had tested positive for anti-Scl-70 antibody are noted in Table 2. The number of cases per laboratory and the laboratories' assay methods are also shown. The anti-Scl-70 antibody titers and reference ranges for each patient are shown in Appendix A.

In three cases the records available to us showed the anti-Scl-70 antibody result but not the laboratory that performed the testing.

30 of 48 patients (62.5%) tested positive for anti-Scl-70 antibody at Quest Diagnostics or LabCorp, commonly used commercial laboratories in Texas and surrounding states that make up most of our clinic's referral base. The majority of positive anti-Scl-70 antibody results were generated by multiplex flow immunoassay (sometimes referred to as multiplex bead assay or multiple bead assay). Links to the companies' websites with anti-Scl-70 antibody testing method information are included in Appendix B. Based on information available on the websites and companies' responses to our inquiries, none of the listed companies offers anti-Scl-70 antibody testing by immunodiffusion. Of note, some companies offer multiple methods of testing depending on the test code that is used (examples are noted in Appendix B). LabCorp offers two options for anti-Scl-70 antibody testing, one via multiplex flow immunoassay and the other via ELISA. The latter option has a reflex to confirmatory testing; the method of the confirmatory test was not noted on LabCorp's website, but through personal correspondence LabCorp indicated that the confirmatory test is currently performed by an electrochemiluminescence immunoassay. Based on the reported titers and reference ranges included in our patients' laboratory reports, we infer that all 11 of the patients in this case series who tested positive at LabCorp were tested using the multiplex flow immunoassay rather than ELISA.

As shown in Appendix C, out of 44 patients whose commercial anti-Scl-70 antibody assay had a reference range of 0–0.9 or <1.0 (the typical reference range reported for the multiplex flow immunoassay), 31 patients (70.5%) had a titer between 1.0 and 4.0, 8 patients (18.2%) had a titer between 4.1 and 8.0, and 5 patients (11.4%) had a titer >8.0. All of the patients with a titer of 8.0 or less tested negative for anti-Scl-70 antibody by immunodiffusion. Two of the five patients with a titer >8.0 tested positive for anti-Scl-70 antibody by immunodiffusion, while three tested negative.

Discussion

In this study, we identified 48 patients who tested positive for anti-Scl-70 antibody in a commercial or local laboratory, but did not have SSc and were negative for anti-Scl-70 antibody by the immunodiffusion method of testing. Many of these patients had no clinical criteria for SSc and/or tested negative for ANA by indirect immunofluorescence. We conclude that some of the anti-Scl-70 antibody results from laboratories commonly utilized in clinical practice are falsely positive.

In a recent retrospective review of clinical data of patients at the University of Utah who had tested positive for anti-Scl-70 antibody by a multiplex bead assay, only 37% were judged to have SSc based on clinical criteria [27]. Another study at the University of Michigan found that only 26.4% of 129 patients who had tested positive for anti-Scl-70 antibody by multiplex flow immunoassay, and only 47.1% of those positive by both multiplex flow immunoassay and ELISA, met clinical criteria for SSc based on retrospective chart review [26]. Most of these patients tested negative for anti-Scl-70 antibody by the immunodiffusion method. 20 out of 21 patients (95.2%) positive by all three assays were judged clinically

to have SSc, showing greater specificity and therefore greater positive predictive value of the immunodiffusion method. To our knowledge, the Michigan study was the first to report direct comparison of the performance of multiplex flow immunoassay methodology to that of immunodiffusion. Regarding the performance of ELISA/EIA methodology, one prior study indicated comparable performance of ELISA compared to immunodiffusion for anti-Scl-70 antibody testing [19], while the Michigan study indicated poorer specificity of ELISA [26]. Variation within the ELISA/EIA format, perhaps due to the source of Scl-70 antigen and/or other technical factors, might contribute to this apparent discrepancy. Indeed, variation in performance was previously observed in a direct comparison of nine unique EIAs for detection of several extractable nuclear antigens (ENAs), including Scl-70 [28]. Most of the false positive anti-Scl-70 antibody results in our case series were generated by multiplex flow immunoassay, notably from a variety of laboratories commonly utilized in clinical practice. Our findings provide direct confirmatory evidence of a false positivity problem in testing methods for anti-Scl-70 antibody in U.S. clinical practice.

We note with disappointment that immunodiffusion testing for anti-Scl-70 antibody is not readily available in clinical practice. In fact, we are not aware of any commercial laboratories in the U.S. that offer it. Thus, clinicians are faced with the burden of positive results from methods with relatively poor specificity, without the recourse of more specific confirmatory testing. This problem is exacerbated by widespread testing for anti-Scl-70 antibody through its inclusion in “reflex” multiplex panels tied to ANA testing offered by some commercial laboratories. Widespread use of a diagnostic test with poor specificity for a relatively rare disease is a setup for a high prevalence of false positive results, especially when applied to patients with a low pre-test probability based on clinical presentation.

The fact that all of the patients in this series with relatively low titer Scl-70 antibody results from multiplex flow immunoassay were negative by immunodiffusion, while two of the five patients with relatively high titer were confirmed as positive by immunodiffusion, lends itself to the hypothesis that the titers from multiplex flow immunoassays might have some predictive significance. This possibility would require further investigation with larger sample sizes. Regardless, we submit that use of a validated, high-specificity assay such as immunodiffusion would be simpler and more reliable for determination of anti-Scl-70 antibody than any attempt to stratify titers from a poor-specificity assay.

The negative impacts of false positive anti-Scl-70 antibody results on patients are difficult to quantify, but some inferences can be made from the cases presented here. By definition, every patient in this study underwent evaluation at a scleroderma specialty clinic, with the associated co-pay and travel expenses as well as time commitment. While some patients had appropriate indications for rheumatology evaluation (for example Raynaud’s phenomenon, positive ANA), others had no apparent reason for referral to a scleroderma specialty center other than the commercially positive anti-Scl-70 antibody result. A considerable number of patients with false positive anti-Scl-70 antibody results had also undergone echocardiograms and/or chest CTs, exposing them to additional costs and, in the case of chest CTs, radiation. These screening studies are appropriate in the setting of clinical suspicion for early SSc or SSc sine scleroderma, as they could allow for early identification of internal organ involvement. However, in most of the cases presented here, the patients who underwent

chest CTs and/or echocardiograms had minimal or no clinical criteria for SSc. Presumably, clinical suspicion of SSc would have been low in these patients were it not for the anti-Scl-70 antibody results from commercial or local laboratories, which turned out to be falsely positive based on our immunodiffusion results. We acknowledge that echocardiograms and chest CT's could have been ordered for other, appropriate indications like respiratory symptoms, and we cannot determine the extent to which the anti-Scl-70 antibody results influenced these decisions. Anecdotally, it is clear to us that many patients experienced angst and confusion about their positive anti-Scl-70 antibody result, and in some cases their perceived diagnosis of SSc. These responses are understandable given the chronic, incurable nature of SSc and its impact on quality of life and mortality. Better awareness of the propensity of commercial assays to produce false positive results and earlier testing for anti-Scl-70 antibody by a specific method like immunodiffusion could have allowed unnecessary diagnostic evaluations and stresses to be avoided.

Our study has several strengths. Each patient was evaluated at their initial and follow up clinic visits by a rheumatologist with experience in the diagnosis of SSc. All immunodiffusion testing was performed by a uniform protocol with interpretation by a single reader (JC), avoiding potential technical or inter-reader variations. The length of the study, relatively large number of cases, broad referral base in Texas and surrounding states, and diverse commercial or local anti-Scl-70 antibody testing locations suggest that our results are generalizable.

Our study also has limitations. Some of the patients who tested negative for anti-Scl-70 antibody by immunodiffusion did not have follow up clinic visits, so could not be evaluated for development of SSc over time. Since we relied on records of chest CTs and echocardiograms that had been performed previously but did not order new diagnostic tests for this research study, many patients were not screened for ILD or pulmonary hypertension and therefore could not be assessed for these SSc criteria. These concerns are counterbalanced to some extent by the fact that many of the patients had no clinical criteria for SSc at the time of their evaluation. Our study design lacked systematic comparisons of anti-Scl-70 antibody testing methods, precluding determination of the sensitivity or specificity of the testing methods involved.

Conclusion

This series of cases highlights the propensity of widely used anti-Scl-70 antibody testing methods to produce false positive results, and some of their potential negative consequences, in clinical practice. We propose that availability of immunodiffusion testing for anti-Scl-70 antibody in clinical practice would directly benefit clinicians and patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Sam Theodore and Pat Gonzales for assistance enrolling patients, drawing blood, and obtaining outside medical records, and Hau Pham and Kenneth Pham for assistance in the laboratory.

Funding

SA is supported by a grant from the National Institutes of Health (RO1AR073284). BS was supported by a grant from the Arthritis National Research Foundation.

Declaration of Competing Interest

SA has received grant support from the NIH, Department of Defense, Scleroderma Research Foundation, Momenta, Boehringer Ingelheim, and Janssen, consulting fees from Boehringer Ingelheim, Corbus, Novartis, CSL Behring, Abbvie, and AstraZeneca, payments or honoraria from Integrity CE and the North Carolina Rheumatology Association, and serves as an unpaid volunteer in leadership roles in the Scleroderma Clinical Trials Consortium and the Scleroderma Foundation Medical Advisory Board. MDM has received personal fees from Actelion Pharma, support to UTHSC—H from Mitsubishi-Tanabe, Boehringer Ingelheim, EICOS, Corbus, and Horizon as a clinical trials investigator, royalties from Oxford University Press, Springer, and BMJ Publishing, consulting fees from Mitsubishi-Tanabe, Boehringer Ingelheim, and Eicos, and payment for lectures from Medtelligence. BHL, JC, RT, MAL, BJ, and BS have no competing interests to declare.

Abbreviations:

SSc	systemic sclerosis
ANA	antinuclear antibody
ILD	interstitial lung disease
ELISA	enzyme-linked immunosorbent assay
ACR	American College of Rheumatology
EULAR	European League Against Rheumatism

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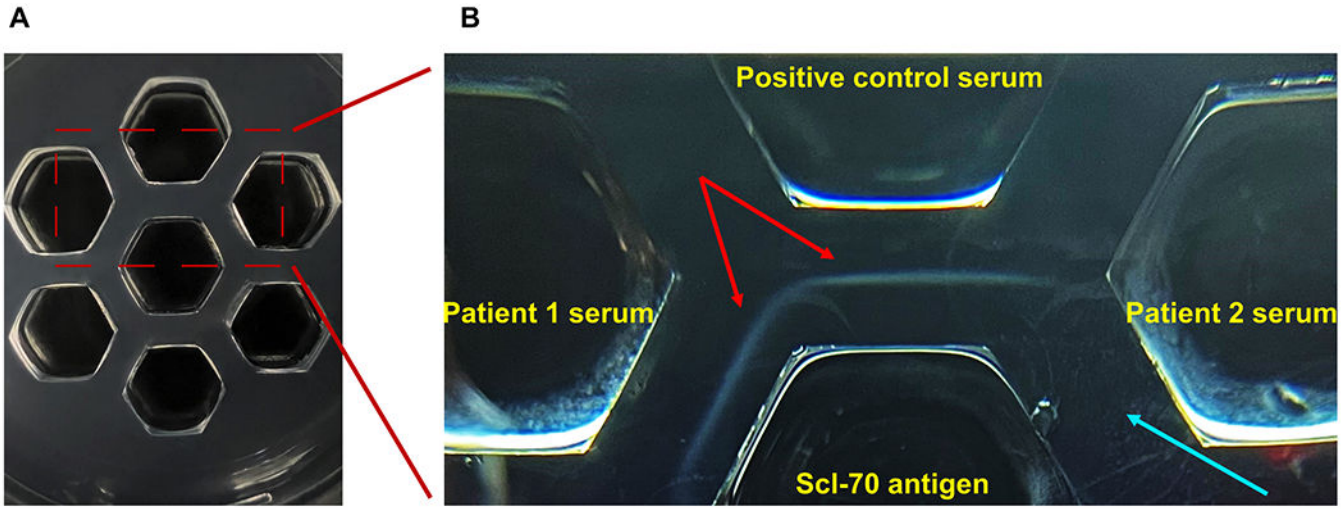

Fig. 1.

Anti-Scl-70 (Topoisomerase I) antibody determination by immunodiffusion. (A) Gel plate used for double immunodiffusion assay. To run the assay, Scl-70 antigen is loaded into the center well. An Scl-70 antibody positive control serum sample is loaded into the wells at the top and bottom. Patient serum samples are loaded in the wells on the left and right. After three days, the gel is examined for precipitin lines. (B) Magnified view of the highlighted section of the gel plate from (A) three days after sample loading. The center well from (A), containing Scl-70 antigen, is shown at the bottom, and the Scl-70 antibody positive control sample is shown at the top. The wells shown at the left and right contained serum samples from two unique patients. Precipitin lines (indicated by red arrows) between the Scl-70 antigen well and the wells at the top (positive control sample) and left (Patient 1 serum) indicate positivity for anti-Scl-70 antibody in these samples. The absence of a precipitin line between the Scl-70 antigen well and patient 2 serum (indicated by the blue arrow) indicates negativity for anti-Scl-70 antibody in this patient.

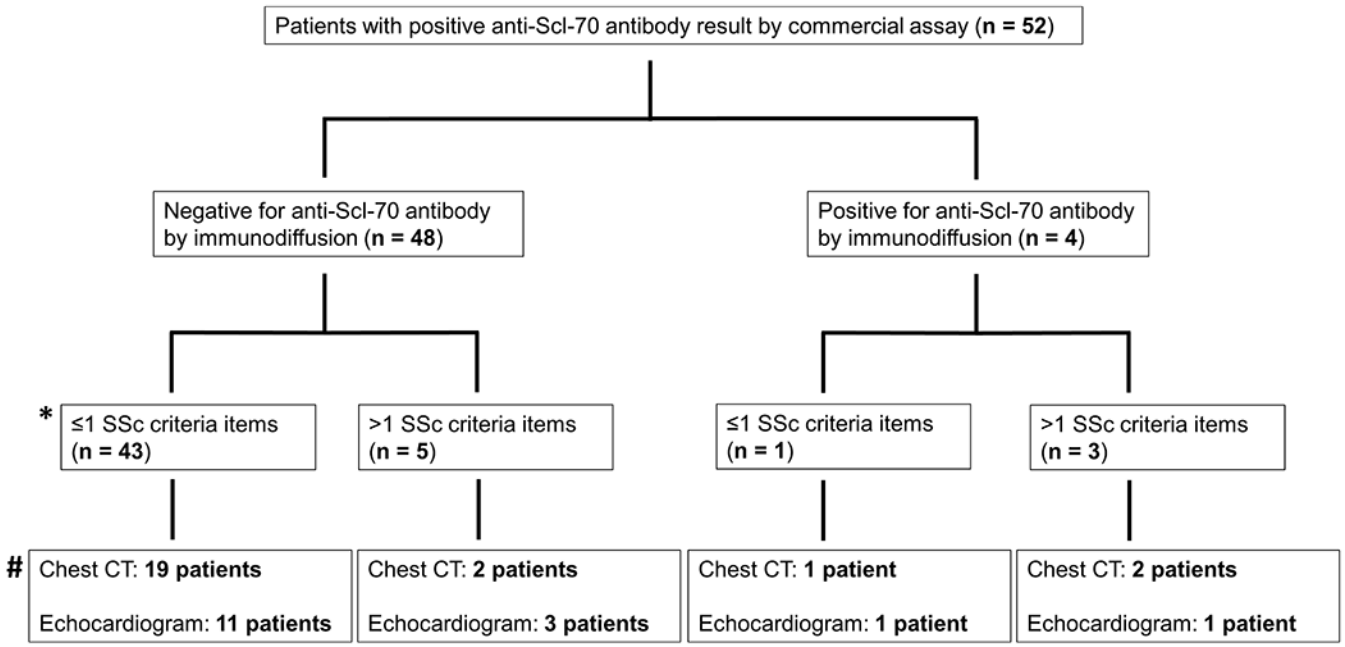

Fig. 2.

Flow diagram showing numbers of patients who underwent chest CT and/or echocardiogram within patient subgroups, by Scl-70 immunodiffusion results and number of ACR/EULAR systemic sclerosis classification criteria items. *Commercial Scl-70 antibody results were not counted towards the criteria items, as noted in the Methods section. #Refers to the numbers of patients who underwent chest CT and/or echocardiogram prior to evaluation in our clinic.

Table 1

Demographics and clinical features.

Demographics and clinical features	Negative for anti-Scl-70 antibody by immunodiffusion (*n* = 48)	Positive for anti-Scl-70 antibody by immunodiffusion (*n* = 4)
Age in years, mean (SD)	45.5 (13.9)	41.3 (3.0)
Female, n (%)	44 (91.7)	3 (75)
History of Raynaud's prior to initial visit, n (%)	15 (31.3)	2 (50)
Points in 2013 ACR/EULAR SSc classification criteria at initial visit, median (min, max) *	0 (0, 7)	4.5 (0, 8)
Positive ANA by indirect immunofluorescence, n (%)	30 (62.5)	4 (100)
Underwent chest CT, n (%)	21 (43.8)	3 (75)
Underwent echocardiogram, n (%)	14 (29.2)	2 (50)
Follow up visit, n (%)	19 (39.6)	4 (100)
Classifiable SSc at follow up visit, n (%)#	0 (0)	3 (75)

*Not including commercial anti-Scl-70 antibody result.

#of those who had at least one follow up visit.

Table 2

Laboratories that produced positive anti-Scl-70 antibody results.

Laboratory	Number of cases, n (%)	Assay Method
Quest Diagnostics	19 (39.6)	multiplex flow immunoassay
LabCorp	11 (22.9)	multiplex flow immunoassay
Clinical Pathology Laboratories*	6 (12.5)	multiplex flow immunoassay (2 patients)
Mayo Clinic Laboratories	1 (2.1)	unclear method (4 patients) *multiplex flow immunoassay
PathGroup Labs	1 (2.1)	multiplex flow immunoassay
The Pathology Laboratory (APMC)	1 (2.1)	EIA
Local clinics or hospitals	6 (12.5)	Not determined
Not determined	3 (6.3)	

* 4 patients' results included titers and a reference range different than that typical of multiplex flow immunoassay. The method of testing for these 4 patients could not be determined from the patient laboratory reports, the company website, or personal inquiries to the company.

EIA: enzyme immunoassay.